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Enhancement in corneal permeability of riboflavin using calcium sequestering compounds

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ABSTRACT

Ethylenediaminetetraacetic acid, ethylenediamine-*N*,*N*′-disuccinic acid and ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid are polyaminocarboxylic acids that are able to sequester metal ions. Calcium is implicated in maintenance of intercellular matrix, zonula occludens (tight junctions) and zonula adherens of epithelium and endothelium cells. Corneal epithelium is impervious to many aqueous formulations due to it being lipophilic, whereby transcellular drug transit is resisted, whilst tight junctions restrict access via the paracellular route. Research has shown that integrity of tight junctions breaks down through loss of Ca^{2+} for endothelial and epithelial cells. This study investigates different Ca^{2+} sequestering compounds and their effect on corneal permeability of riboflavin at physiological pH. Riboflavin is a topically administered ocular drug applied during UV-induced corneal cross-linking for the treatment of keratoconus.

1. Introduction

Administering drugs to treat ocular conditions is confronted with many challenges despite easy access to the eye. Drug delivery is hampered by blink action, washout by tears, mechanisms of nasolacrimal drainage and poor permeability of the cornea (Gilhotra et al., 2011; Kumaran et al., 2010). Once the dosage form is applied there is only a short time where the medication is in contact with the eye, during this time drugs intended for intraocular tissue have to penetrate the cornea (Washington et al., 2001). A small proportion of topically applied drugs are absorbed into the cornea, up to 5% but often much less (Jarvinen and Jarvinen, 1996). Most of the instilled dose is systemically absorbed by the nasolacrimal duct and conjunctiva (Wilson et al., 2001). The cornea is a multilayered structure consisting of five layers: epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium. The epithelium is a lipophilic layer of around 10% of the total corneal thickness, offering a barrier of around 90% to hydrophilic drugs and 10% to hydrophobic drugs (Washington et al., 2001; Wilson et al., 2001). The Bowman's membrane forms a thin transitional layer towards the stroma, which is the main section at ~90% of the total corneal thickness with hydrophilic gel properties comprised of collagen fibrils, other proteins and mucopolysaccharides. There exists an aqueous environment between collagen fibres, and this contributes to the barrier function that the stroma offers against lipophilic drugs. The next layer is the Descemet's membrane, a tough but thin, homogenous layer deposited by the endothelium, a single layer of cells important in maintaining optimal corneal hydration (Washington et al., 2001; Wilson et al., 2001). Animal eyes are often used as models when researching ocular drug delivery because they share many of the features present in human eyes. Bovine eyes prove to be recognized models, and these were employed in this investigation (Chandran et al., 2008; Loch et al., 2012).

Corneal epithelium itself has further layering consisting of a basal layer of newly formed columnar cells; these are pushed

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progressively towards the surface as new cells develop; at this stage they are polyhedral shaped. Finally there is the superficial layer of polygonal shaped squamous cells, these cells have microvilli to aid mucus adhesion, which further aids adherence of the tear film. Superficial epithelial cells are surrounded by Ca^{2+} dependent cell binding sites, zonula occludens, otherwise known as tight junctions, immediately below these are zonula adherens, and towards the base of the cells there are spot like contact zones known as desmosomes. The combination of these cell membrane adherent regions and the lipophilicity of epithelia provide a very effective barrier function that protects the eye from ingress of alien material (Jain-Vakkalagadda et al., 2004; Kaur and Batra, 2007).

The effectiveness of the epithelium barrier is dependent on an undetermined concentration of Ca^{2+} ions being available to the plasma membrane. Calcium sequestering compounds are effective at disrupting corneal epithelia by extracting Ca^{2+} ions (Kaur and Smitha, 2002).

Polyaminocarboxylic acids are a class of synthetic compounds with metal ion sequestering properties. Ethylenediaminetetraacetic acid (EDTA) is an effective calcium chelator which is included in many ocular drug formulations as a preservative and stabiliser (Freeman and Kahook, 2009; Grass et al., 1985); however there are concerns regarding its accumulative toxicity towards intraocular structures when used in the longer term. The present study investigates the use of EDTA together with two analogues, ethylenediamine-*N*,*N*′-disuccinic acid (EDDS) and ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) for their effectiveness as penetration enhancers in delivery of riboflavin for the treatment of keratoconus and other corneal disorders (Fang-Sheng et al., 1992; Grass et al., 1985; Meers et al., 2005; Oviedo and Rodriguez, 2003). Around 1 in 2000 of the general population is afflicted with keratoconus, a debilitating ocular condition whereby the cornea degenerates, and the patient's vision becomes seriously compromised. The condition can develop to the stage where penetrating keratoplasty (corneal transplant) becomes necessary (Tuft et al., 1994). Wollensak et al. (2003) published their work on development of a novel procedure for treating keratoconus by ultraviolet A induced riboflavin–collagen cross-linking. This procedure has now become a common practice for this and other corneal degenerative conditions. However, the procedure relies on physical abrasion of the epithelium under anaesthesia to allow corneal permeation of riboflavin solution into the stroma. Rupenthal et al. (2011) report on what they term 'the corneal wound healing cascade' initiated when corneal epithelia sustain injury, cytokines are released leading to keratocyte apoptosis followed by several more events before eventual return to normality. Development of a means to deliver this drug without resorting to epithelial debridement would be a major improvement on the procedure.

The present study investigates the effects of EDTA, EGTA and EDDS calcium sequestering compounds on corneal permeability of riboflavin after first determining their Ca^{2+} binding ability using isothermal titration calorimetry (ITC). We also explored corneal integrity after exposure to these solutions. Analysis of these solutions before and after corneal exposure was carried out using atomic absorption spectrometry (AAS), and it was established that they are able to extract calcium ions from corneal epithelial membranes. Corneal barrier function was evaluated using trans-epithelial electrical resistance analysis (TEER) to determine changes in electrical resistance of the cornea after exposure to Ca^{2+} chelating solutions. A reduction in electrical resistance correlates to increased permeability, supporting the hypothesis that tight junctions are dependent on Ca^{2+} availability (Kikuchi et al., 2005). Permeability studies using Franz diffusion cells (FDC) confirmed that polyaminocarboxylic acids enhance corneal permeability of riboflavin compared to the drug dissolved in PBS. Examination using microscopy has shown that the histology of the epithelial barrier was altered when they were exposed to these compounds.

2. Materials and methods

2.1. Materials

Ethylenediaminetetraacetic acid (EDTA), ethylenediamine-*N*,*N*′-disuccinic acid (EDDS), ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2-(*N*-morpholino) ethanesulfonic acid (MES), riboflavin, sodium hexane-1-sulfonate monohydrate and glacial acetic acid were purchased from Sigma–Aldrich (Gillingham, UK). 1000 ppm calcium calibration standard in nitric acid, 10% w/v lanthanum chloride, calcium chloride, sodium chloride, potassium chloride, sodium phosphate, potassium dihydrogen phosphate, sodium hydroxide, hydrochloric acid, optimal cutting temperature compound (OCT) and HPLC grade ethanol were obtained from Fischer Scientific (Hemel Hempstead, UK). Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Vector Laboratories Ltd. (Peterborough, UK). MilliQ ultrapure water ($18\,m\Omega\,cm^{-1}$) was used for all aqueous solutions. All materials were used as supplied without modification.

2.2. Preparation of buffer solutions

Isotonic PBS was prepared in-house and was adjusted to pH 7.4 ± 0.2 using 0.1 M NaOH solution (Roskams and Rodgers, 2002). Ion-pair buffer for HPLC analysis was prepared using sodium hexane-1-sulfonate monohydrate (0.1 M) adjusted to pH 3.0 ± 0.2 using glacial acetic acid (Anyakora et al., 2008). For isothermal titration calorimetry, MES (10 mM) buffer was prepared and adjusted to pH 7.4 ± 0.2 using 1 M NaOH solution.

2.3. Isothermal titration calorimetry

ITC analysis was used to determine the Ca^{2+} binding properties of EDTA, EGTA and EDDS using Microcal model ITC200 calorimeter (cell volume = 200 μL), with ITC200 software version 1.24.2 (MicroCal Inc., USA). Data acquisition and graphical analysis was achieved using Origin 7 SR4, v7.0552 software (OriginLab Corporation, USA). Water was placed in the reference cell and $10\,\mu Cal\,s^{-1}$ selected for reference power. 0.4 mM solutions of EDTA, EGTA and EDDS in 10 mM MES buffer (pH 7.4 ± 0.2) were titrated by $CaCl_2$ in 10 mM MES buffer (pH 7.4 ± 0.2). Calcium chloride titrant at 6 mM was used for EDTA and EGTA, a higher concentration at 25 mM was used for EDDS in order to reach saturation. A method with an initial injection of 0.4 μL followed by 15 injections of 2 μL with spacing of 150 s; analysis was carried out at 25 °C.

2.4. Atomic absorption spectroscopy (AAS)

Calcium ion analysis was conducted using a novAA 350 system with WinAAS software, version 4.5.0 (Analytik JENA, Germany). Following the method for calcium analysis published in '*Atomic Absorption Data Book*', briefly, 50 mm stoichiometric air/acetylene flame, 422.7 nm wavelength, ultrapure water ($18\,m\Omega\,cm^{-1}$) as the carrier (Whiteside and Milner, 1983). Quantitation achieved by reference to a calibration curve produced from Ca^{2+} standards in ultrapure water incorporating 5% w/v lanthanum chloride as a releasing agent. Standards were prepared at Ca^{2+} concentrations ranging from 0.1 to 10.0 ppm ($r^2 = 0.9921$). Samples are diluted for analysis by a factor of 100, therefore a lower concentration of aqueous $LaCl_3$ at 0.25% w/v is used for their preparation.

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